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Folate-Mediated Targeting of Therapeutic and Imaging Agents to Cancers

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ABSTRACT: The vitamin folic acid (FA) enters cells either through a carrier protein, termed the reduced folate carrier, or via receptor-mediated endocytosis facilitated by the folate receptor (FR). Because folate–drug conjugates are not substrates of the former, they penetrate cells exclusively via FR-mediated endocytosis. When FA is covalently linked via its γ-carboxyl to a drug or imaging agent, FR binding affinity ($K_D \sim 10^{-10}$M) is not measurably compromised, and endocytosis proceeds relatively unhindered, promoting uptake of the attached drug/imaging agent by the FR-expressing cell. Because FRs are significantly overexpressed on a large fraction of human cancer cells (e.g., ovarian, lung, breast, endometrial, renal, colon, and cancers of myeloid hematopoietic cells), this methodology may allow for the selective delivery of a wide range of imaging and therapeutic agents to tumor tissue. Folate-mediated tumor targeting has been exploited to date for delivery of the following molecules and molecular complexes: (i) protein toxins, (ii) low-molecular-weight chemotherapeutic agents, (iii) radioimaging agents, (iv) MRI contrast agents, (v) radiotherapeutic agents, (vi) liposomes with entrapped drugs, (vii) genes, (viii) antisense oligonucleotides, (ix) ribozymes, and (x) immunotherapeutic agents. In virtually all cases, *in vitro* studies demonstrate a significant improvement in potency and/or cancer-cell specificity over the nontargeted form of the same pharmaceutical agent. Where live animal studies have been conducted, they also reveal significant promise.

KEY WORDS: Folate receptor endocytosis, tumor targeting, tumor imaging, cancer chemotherapy, folic acid conjugates.

I. INTRODUCTION

Cellular uptake of folic acid and its reduced cogeners is facilitated by two unrelated proteins: a low-affinity ($K_D \sim 1\ \mu M$–$5\ \mu M$) membrane-spanning protein that catalyzes the passive transport of reduced folates into cells, and a high-affinity ($K_D \sim 100$ pM)

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glycoprotein receptor, termed the folate-binding protein or folate receptor (FR), that preferentially promotes endocytosis of folic acid (FA).[1-6] This latter receptor is actually not a single species, but rather a family of proteins encoded by three distinct genes: FR-α, simultaneously characterized in KB cells,[7] CaCo-2 cells,[8] and placenta[9]; FR-β, originally studied in placenta[10]; and FR-γ (and the truncated FR-γ'), recently detected in malignant hematopoietic cells.[11] These folate-receptor homologues are related by approximately 70% amino-acid sequence identity. FR-α and FR-β are attached to the cell surface by a glycosylphosphatidylinositol (GPI) anchor, while FR-γ is secreted due to lack of an efficient signal for GPI modification.[11] For a more thorough description of the properties of these receptors, the reader is referred to several excellent reviews and primary reports.[1,2,5,6,12-15]

In conventional cancer chemotherapy, a linear increase in the fraction of cells killed often requires an exponential increase in drug dose. However, because of toxic side effects, only limited quantities of chemotherapeutic agents can generally be administered, frequently allowing a small fraction of the more resistant cancer cells to survive. Therefore, the development of techniques that can deliver the maximum dose of administered drug specifically to tumor cells has assumed a high priority. While much research has focused on the use of tumor-specific monoclonal antibodies to target attached drugs to cancer tissues, the large size and frequent immunogenicity of antibodies has severely compromised this approach. In a search for low-molecular-weight ligands that can similarly target drugs with high specificity to cancer cells, one ligand, folic acid, has emerged as an attractive substitute for tumor-selective antibodies. This review summarizes the use of folic acid as a targeting ligand for the specific delivery to cancer cells of protein toxins, imaging agents, low-molecular-weight chemotherapeutic agents, genes, antisense oligonucleotides, enzymes for prodrug therapy, radiotherapeutic agents, liposome-encapsulated drugs, and immunotherapeutic agents. Conjugates of folic acid linked to therapeutic or imaging agents bind to the folate receptor on the tumor cell surfaces and are transported into the cancer cells by receptor-mediated endocytosis (Figure 1).

II. DISTRIBUTION AND EXPRESSION OF FOLATE RECEPTORS

In 1991, a clinically valuable tumor marker was purified from ovarian cancers by two independent groups, and sequence analysis showed that it was the receptor for folic acid.[16] This and homologous receptors have now been shown to be overexpressed on the cell surfaces of a number of different types of human cancers.[16-19] In general, FR-α is upregulated in malignant tissues of epithelial origin such as ovarian carcinoma,[18,20] while FR-β is overexpressed in malignant tissues of nonepithelial origin.[18] In fresh tumor specimens, the folate receptor has been found in >90% of ovarian and other gynecological cancers.[21,22] In another study, approximately 50% of lung carcinomas and 25% of breast cancers were also receptor-positive,[23] and in a third study four out

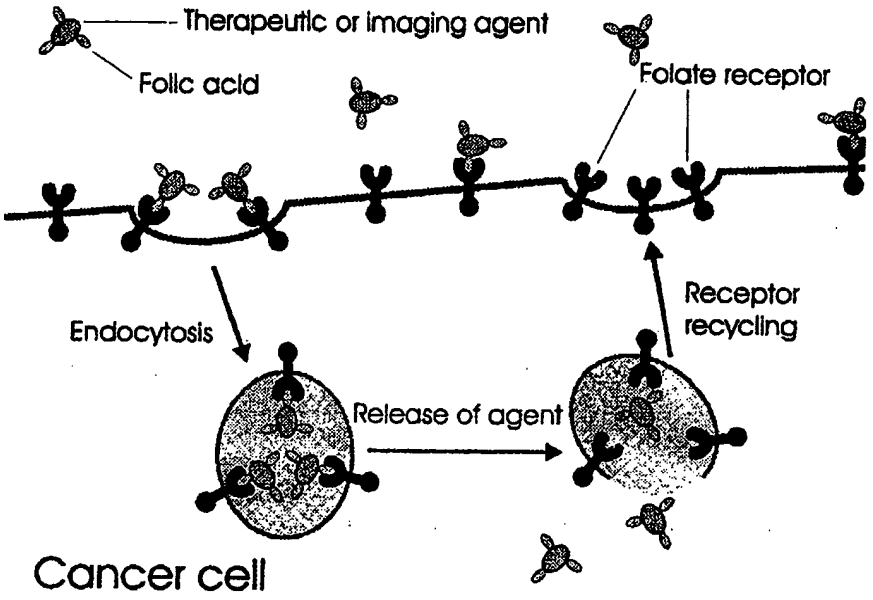

FIGURE 1. Diagrammatic representation of the internalization of folate–drug conjugates by folate receptor–mediated endocytosis.

of six brain tumors were seen to overexpress the FR.[17] Based on data from a number of laboratories, it can be concluded that FR is frequently overexpressed in cancers of the ovary, endometrium, lung, breast, brain, kidney, colon, and hematopoietic cells of the myeloid lineage.[16–25] When levels of FR are compared among a large number of ovarian cancer tissue samples, a strong correlation is observed between folate receptor content and both the grade and histological stage of the tumor.[24] In general, highly dedifferentiated metastatic cancers express considerably more FR than their more localized, low grade counterparts. The FR has also been mapped to a region (11q13) amplified in greater than 20% of tissue samples from breast, head and neck tumors.[26]

Folate receptors have also been detected in normal tissues involved in the retention and uptake of the vitamin. The choroid plexus expresses high levels of the α-isoform; however, the receptor appears to be localized exclusively on the brain side of the blood–brain barrier, and hence it is probably inaccessible to blood-borne folate conjugates.[25,27] The FR has been similarly observed on the intestinal brush border apical membrane surface,[28] but again access to these folate docking sites requires either escape from the bloodstream or oral ingestion of the folate–drug conjugate. Thirdly, the α homologue of the folate receptor is enriched in the proximal tubules of the kidney,[12] where it probably functions to salvage folic acid that escapes in the

filtrate. This receptor is accessible to folate conjugates excreted in the urine[29,30] but is inaccessible to conjugates too large to filter through the kidneys. Finally, the folate receptor (α) is also detected at low levels in a few other normal tissues,[18] but its relative abundance in these tissues is low compared to several cancers, as emphasized by the fact that the receptor has been reliably employed for many years as a tumor marker.[16-25]

While the β isoform of the folate receptor may be present in a number of nonepithelial tissues, its level of expression appears to be at least an order of magnitude lower than that of the α form in the above malignant tissues.[17,18] For example, we have detected the β isoform in CD34$^+$ hematopoietic cells (Reddy, Low, Clapp, Haneline, Srour, and Antony, personal observations), but our assays reveal its concentration to be at least 100-fold lower than the level of FR-α in ovarian cancer cells. Consequently, intravenously administered folate conjugates are measurably internalized only by folate receptor-expressing tumor cells and kidney epithelial cells that encounter the drug during excretion from the body.[29,30]

III. INTERNALIZATION OF FOLATE-CONJUGATED MOLECULES

Free folates have been shown to be taken up by cells via a receptor-mediated pathway. In one model termed potocytosis,[31] folates are proposed to bind receptors clustered around caveolæ, i.e., specialized regions of the cell membrane enriched in cholesterol and other GPI anchored proteins.[32] These caveolæ then invaginate, forming a membrane-tethered compartment within the cytoplasm. An internal proton gradient is then proposed to facilitate release of the bound folate, allowing its movement across the caveolæ membrane by an integral membrane anion carrier.[33] In conflict with this model, however, are data showing that FR exhibit no tendency to collect in caveolæ unless they are crosslinked by bivalent antibodies.[34-36] These reports have also raised the possibility that the endocytosis observed in earlier studies occurs at coated pits rather than caveolæ. Clearly, the matter regarding the internalization route of FR remains unsettled, and future investigations of the pathway will have to exercise unusual precautions to ensure that the tools used to characterize the system do not change it.

Five lines of evidence demonstrate that folate-conjugated molecules enter cells by folate receptor-mediated endocytosis. First, a variety of folate-derivatized proteins have been shown to concentrate inside folate receptor–expressing cells, while identical proteins lacking the folate ligand display no affinity for the same cells.[37] Second, pretreatment of FR positive cells with an excess of free folic acid has been found to competitively inhibit uptake of folate conjugates.[37] Third, occlusion of cell-surface folate receptors with antireceptor antibodies prevents folate-conjugate uptake.[38,39] Fourth, selective release of folate receptors from their membrane anchors by cell-surface digestion with a phosphatidylinositol-specific phospholipase C renders the

treated cells unable to internalize folate conjugates.[38,39] And finally, the affinity and total number of sites for folic-acid binding to cultured cells closely approximates the affinities and total numbers of binding sites of several folate conjugates to the same cell surfaces.[37] Taken together, these data argue that the well-characterized folate receptor is responsible for folate-conjugate uptake by folate receptor–bearing cells.

The intracellular itinerary followed by folic-acid conjugates during uptake by receptor-expressing cells has been partly characterized using folate-derivatized colloidal gold particles (FA–CG) and electron microscopy. The results indicate that FA–CG particles bind to KB cells in or near small membrane pits, probably caveolæ, where they occur in clusters. The role of caveolæ in FA-conjugate endocytosis was more strongly suggested by measuring the amount of internalized FA conjugates in the presence of two caveolæ perturbing agents. Nystatin, a cholesterol-binding drug causes disassembly of caveolæ and the consequent unclustering of FRs,[3,40,41] whereas phorbol-12 myristate acetate (PMA), a protein kinase C activator, directly inhibits the internalization process.[42] Importantly, although neither drug exerts a significant effect on endocytosis at coated pits,[42] internalization of folate conjugates by cancer cells was significantly reduced in the presence of either agent.[43] Very recently, immunofluorescence data have also suggested folate-conjugate uptake at caveolæ.[44] However, none of the above studies have been highly definitive, and so we consider the identity of the port of entry of folate conjugates still unresolved.

Studies utilizing CG-labeled anti-FBP antibody in MA 104 cells have suggested that the FR remains permanently associated with caveolæ and does not internalize with transport vesicles in an endocytic pathway.[40,41] However, FA–CG particles were clearly found to proceed from primary endosomes to multivesicular bodies (MVBs) and then on to tubular endosomes.[45] At later time points, many conjugates were still found in the MVBs, while others were transported to secondary lysosomes or deposited free in the cytoplasm. While it is possible that the large fraction of free cytoplasmic conjugates might have arisen from nonspecific breakdown of an organellar membrane enclosing the FA–CG rather than any programmed release during the trafficking pathway, the data unequivocally demonstrate that multivalent folate conjugates follow an endocytic route into the cell and do not remain attached to the cell surface, as stated in the potocytosis model.

To compare the intracellular itinerary of FA–CG with that of a protein taken up via the clathrin coated pit pathway, transferrin–colloidal gold particles (25 nm) were mixed with FA–CG particles (5 nm) and their uptake was examined by electron microscopy. Following endocytosis at their respective cell-surface assemblies, the transferrin–gold conjugates and the FA–CG were soon found to colocalize in the same multivesicular bodies. These data would suggest that at least some folate conjugates can merge into the same endocytic pathway utilized by ligands entering at clathrin coated pits.[45]

To characterize the pH changes that FA conjugates experience during intracellular trafficking, the pH values of individual endosomal compartments containing endocytosed FA conjugates in KB cells were determined.[43] For this purpose, the fluo-

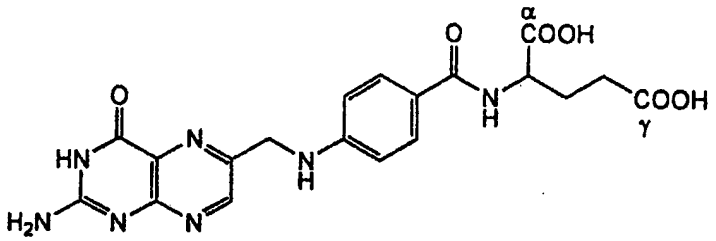

FIGURE 2. Structure of folic acid (FA) with the α and γ carboxyl groups labeled.

rescence ratio of the folate conjugate of a pH-sensitive dye (DM–NERF, pKa 5.5) and a pH insensitive dye (Texas Red) was used to calculate compartmental pH. Although the pH values of individual endosomes varied considerably (4.3 to 6.9), the average pH of ~5 was found to be similar to that seen in both the coated pit and caveolæ endocytosis pathway.[31,43]

IV. METHODS FOR COUPLING FA TO LIGANDS

FA is most easily covalently attached to a ligand via either its α or γ carboxylic-acid moiety (Figure 2). However, it has been shown that the α-carboxyl derivatives are not avidly recognized by the folate receptor, whereas the γ derivatives display the same affinity as free folic acid.[46,47] (Figure 2) Where multiple folates can be attached to a pharmaceutical agent such as a protein or liposome, carboxyl-group selectivity may not be overly important. Thus, random carbodiimide-mediated conjugation of three to five folates per protein should assure that at least one folate is attached via the γ-linkage, especially since this site of derivatization is sterically favored.[37,38] However, where a single folate will be relied on for targeting a drug molecule (e.g., in the case of low-molecular-weight drugs and imaging agents), selective derivatization at the γ-carboxyl group may be required,[46,47] (see Ladino et al.[44] for an opposing view). In the paragraphs below, we will briefly summarize the methods for each type of folate derivation.

FA is a light-sensitive organic molecule with several notable physical properties. The unionized form of FA has limited solubility in water (10 mg/L at 0°C, 500 mg/L at 100°C), while the disodium salt displays higher solubility (15 g/L at 0°C).[48] In aqueous solution, folic acid and some of its conjugates tend to associate into hydrogen-bonded planar tetramers, and these tetramers may further stack to form octamers, dodecamers, etc. that yield separate peaks when analyzed by HPLC.[49] Folic acid is also poorly soluble in most organic solvents except dimethylsulfoxide (DMSO) and di-

methyl formamide (DMF). When dried in a vacuum desiccator under reduced pressure, folic acid retains 5% to 11% moisture, which corresponds approximately to 1 to 2.5 moles of water per mole of folic acid. The water is lost, however, on drying under vacuum at temperatures over 140°C.[48] Finally, folic acid can be destroyed by extended exposure to high temperatures or strongly alkaline solutions. All these properties must be kept in mind when preparing and purifying folate conjugates.

A. EDC Method

The EDC method is the preferred method for coupling multiple FA moieties to the free amines of macromolecules such as proteins,[50] antibodies,[51,52] and amine-containing polymers[43] that are soluble in aqueous medium. In this procedure, FA is dissolved in anhydrous DMSO and mixed with a five-fold molar excess of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) for one hour. The macromolecule is then dissolved in a phosphate-boric acid buffer, pH 8.5, and incubated with a 10- to 20-fold molar excess of the activated vitamin for 1-16 h. In the case of poly-lysine labeling, however, the reaction is successfully conducted in 20 mM sodium phosphate buffer, pH 4.5.[53] Unreacted FA can then be separated from the labeled macromolecule using a Sephadex G-25 column equilibrated in phosphate-buffered saline, pH 7.4. Adjustment of the reaction mixture to pH 9 has been found to be useful for complete removal of free FA from the FA conjugate during gel separation.[43] To quantitatively eliminate unbound FA, the conjugate can also be extensively dialyzed against a neutral pH buffer. The extent of FA conjugation can be determined spectrophotometrically at 363 nm (FA: E_m = 6197 M^{-1} in phosphate buffered saline, pH 7.4). In most cases, a single folic acid is adequate to successfully target an attached macromolecule; however, since this method yields a mixture of both the γ- and α-folate derivatives, it is advisable to aim for a folate-to-macromolecule ratio of 3 to 5 to assure that at least one "active folate" is present on each macromolecule.

B. DCC Method

The DCC method can be used for conjugating FA to low-molecular-weight organic molecules like deferoxamine mesylate[54] or polyoxyethylene-bis-amine[55] that are soluble in organic solvents like DMSO. Equimolar quantities of FA and dicyclohexylcarbodiimide (DCC) plus trace amounts of pyridine are mixed with the ligand and stirred overnight. The insoluble byproduct, dicyclohexylurea, is then separated by centrifugation. If possible, the conjugate can be precipitated with acetone and washed with diethyl ether. The product can then be purified by anion exchange chromatography on a DEAE-trisacryl Sepharose column using 50 mM ammonium bicarbonate buffer as an eluant.[46] As above, the DCC method yields a mixture of γ- and α-folate derivatives.

C. Methyl-Folate Method

In the methyl-folate method (Figure 3), folic acid (1) is first treated with excess trifluoroacetic anhydride and then hydrolyzed to give N^{10}-(trifluroacetyl) pyrofolic acid (2). Reaction of this intermediate (2) with hydrazine yields pteroyl hydrazide (3), which is then oxidized to pteroyl azide (4). Pteroyl azide is then reacted with methyl glutamate in the presence of tetramethyl guanidine to afford exclusively γ-methylfolate (5), a γ-activated folic acid. The γ-methylfolate can be reacted with a free amine-containing molecule to give a γ-specific conjugate. For example, reaction of γ-methylfolate with ethylene diamine generates the γ-conjugate in 87% yield,[56] the remainder being unreacted starting material and other unidentified byproducts.

V. CYTOTOXICITY STUDIES OF FOLATE-LINKED TOXINS

Antibody-linked toxins have been explored as tumor-selective killing agents in a number of clinical and preclinical studies.[57] Although several recent constructs have shown considerable promise, in many cases the antibody–toxin conjugates have been compromised by their (i) large size and consequent reduced penetration of the tumor, (ii) elicitation of a host immune response (except when humanized antibodies are used), (iii) diminution of antigen affinity due to steric hindrance from the attached toxin, (iv) expense of expression and purification of the monoclonal antibody, (v) inefficiency of site-specific conjugation of the toxin to the antibody, and (vi) poor tumor selectivity.[57,58] While alternative peptide and cytokine ligands have remedied some of the above inadequacies, these ligands are also hampered by problems of their own. In contrast, except for the development of an immune response against the toxin, folic-acid–toxin conjugates may exhibit few of the handicaps that encumber the above larger ligands. However, only a few studies have been conducted with folate–toxin conjugates, and so their usefulness as chemotherapeutic agents remains to be fully characterized.

FA–toxin conjugates were initially constructed using a plant vacuolar protein, momordin,[38] and a truncated recombinant bacterial protein, pseudomonas exotoxin A (PE).[46] Momordin functions by inhibiting protein synthesis through modification of the 60S ribosomal subunit. PE mediates the ADP-ribosylation of elongation factor 2, which arrests protein synthesis and causes cell death. Importantly, momordin and the truncated recombinant PE are biologically inactive because they lack a binding domain that would normally facilitate their binding and entry into cells.

FA–momordin conjugate was found to inhibit protein synthesis in a cell-free reticulocyte lysate to the same extent as the unmodified toxin (IC_{50} ~1 nM).[38] To assess the contribution of folate ligation to FA–momordin's toxicity to whole cells, the cytotoxicity of FA–momordin was compared to that of unmodified momordin by determining the amount of radiolabeled leucine incorporated in KB (a human oral carci-

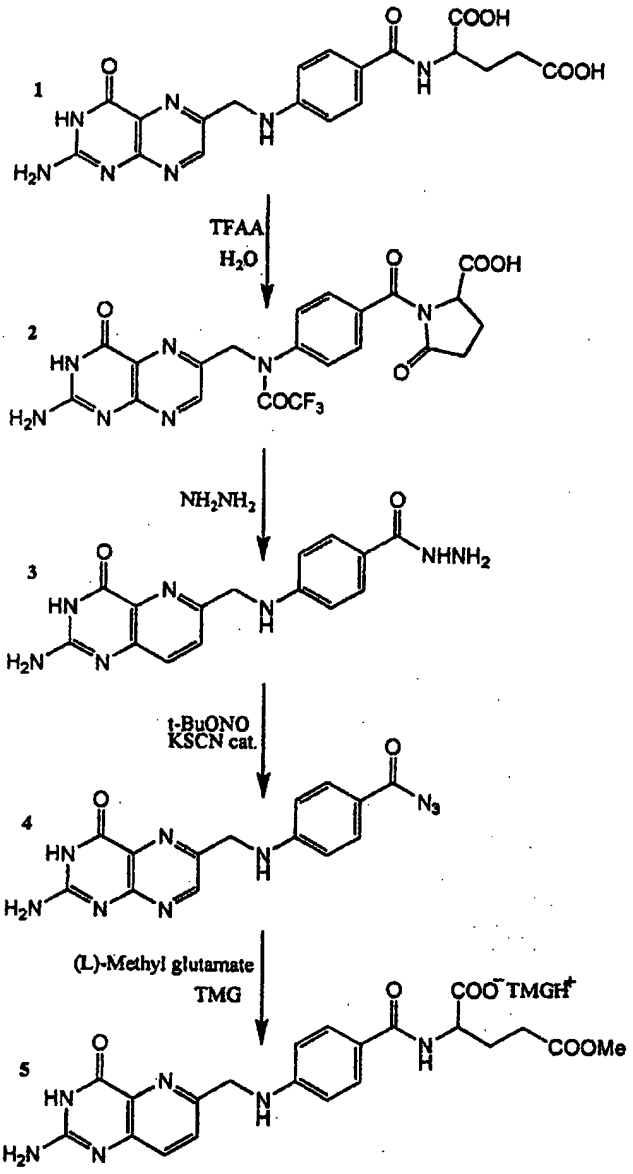

FIGURE 3. Synthetic scheme of the γ-methyl folate method. Abbreviations stand for the following: TFAA, trifluoroacetic anhydride; t-BuONO, *tert*-butyl nitrite; KSCN, potassium thiocyanate; TMG, tetramethyl guanidine.

noma) and HeLa cells (a human cervical carcinoma) after incubation with these toxins.[38] The IC_{50} of FA-momordin ranged between 0.6 and 1.6 nM for both KB and HeLa cells, but was $>10^{-6}$ M for nontargeted momordin.[38] The amount of internalized FA-momordin neared saturation by 5 h incubation, while inhibition of protein synthesis began within 8 h and increased until the final time point of 48 h. The latency period between toxin uptake and interruption of protein synthesis may involve the trafficking time of the toxin along its endosomal pathway prior to entry into the cytoplasm, as seen with other toxin conjugates like human chorionic gonadotropin-ricin A.[59]

To test whether folate receptor-mediated endocytosis might selectively target toxins to tumor cells, a mixed cell culture containing normal and transformed cells was established. Co-cultures of HeLa and WI38 cells (a normal human embryonic lung fibroblast), as well as KB and Hs67 cells (a normal human thymic fibroblast), were pulsed with FA-momordin or underivatized momordin for 20 minutes and then grown for the indicated times in the absence of toxin (Figure 4). In co-cultures pulsed with momordin, the more aggressive transformed cells dominated the co-culture, while in co-cultures pulsed with FA-momordin, the transformed cells were selectively eliminated, with the normal cells achieving a near confluent state. Analysis of folate receptor number showed that the KB and HeLa cells associated with 280 and 160 pmol FA conjugate/mg of cellular protein, while the WI38 and Hs67 cells did not bind any detectable toxin. This correlation indicated that the receptor density might be a good predictor of FA-toxin sensitivity.[50]

Since FA conjugates were observed to remain in endosomal compartments for extended periods after cellular uptake, bacterial toxins with and without their translocation domains were compared for relative toxicity. HeLa cells were pulsed with either FA-momordin lacking a translocation domain or FA-LysPE38, an engineered form of pseudomonas exotoxin containing a translocation domain. FA-momordin required 19 h for 50% inhibition of total cellular protein synthesis and 48 h for complete inhibition. In contrast, FA-LysPE38 required 3 h for 50% inhibition and 12 h for complete inhibition. Since both toxins interrupt protein synthesis immediately upon entry into the cytoplasm, the accelerated rate of protein synthesis inhibition of the FA-LysPE38 can probably be attributed to its ability to facilitate its own escape from an intracellular compartment. The IC_{50} value of FA-momordin was 1.1×10^{-9} M and that of FA-LysPE38 was 1.1×10^{-10} M. This 10-fold increase in potency could also be related to the enhanced entry of FA-LysPE38 into the cytoplasm[46]; however, differences in intrinsic toxicity could also be responsible.

To confirm the importance of a mechanism of endosomal escape for toxin potency, a comparison of the cytotoxicities of translocation-competent and translocation-incompetent forms of the same truncated pseudomonas exotoxin conjugate was made. CysPE35, a shorter form of pseudomonas exotoxin, contains a single free cysteine (Cys^{287}) that must be reduced for translocation to occur. Consequently, conjugation of ligands (e.g., folic acid) to Cys^{287} through a stable thioether linkage inhibits the translocation ability of the toxin, while attachment via a reducable disulfide bond allows restoration of the free cysteine following entry into reducing compartments

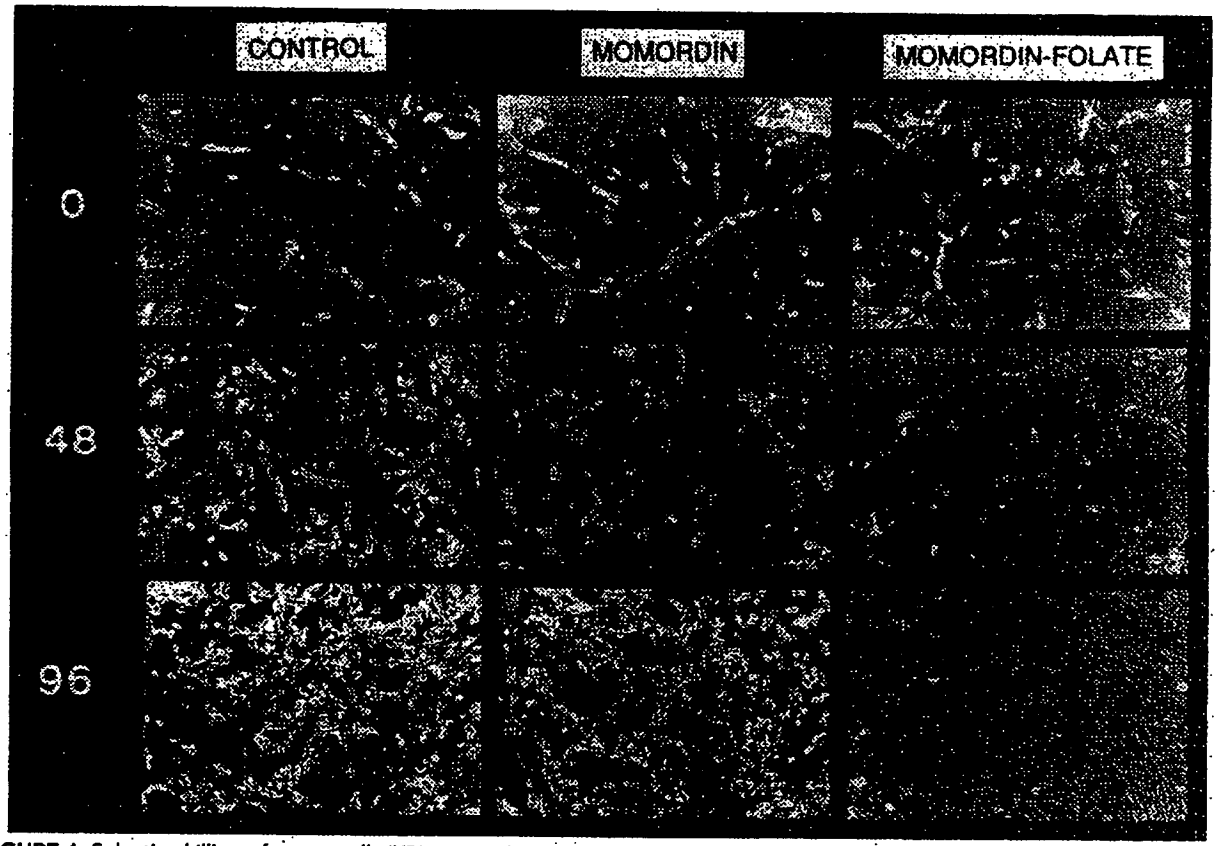

FIGURE 4. Selective killing of tumor cells (KB) in co-culture with normal cells (Hs67). KB/Hs67 cell co-cultures were treated with 1 × 10^{-7} M momordin or momordin–folate conjugate for 20 min or left untreated (control) and then grown for the indicated times in the absence of toxin. Cells were examined under a phase contrast microscope and photographed. (From Leamon et al.[50] by copyright permission of Harwood Publishers.)

TABLE 1
Cell Uptake Capacity of FA–Ribonuclease and their Corresponding Sensitivities to FA–Toxin Conjugates

Cell line	FA–protein uptake (pmol/mg cell protein)[a]	IC_{50}(M) FA–momordin[b]	IC_{50}(M) FA–LysPE38[b]	IC_{50}(M) Ricin[c]
FDKB	280 ± 3.0	1.0×10^{-9}	1.0×10^{-10}	ND
FDHeLa	172 ± 4.0	1.1×10^{-9}	1.1×10^{-10}	ND
2008–FBP	72 ± 6.0	1.0×10^{-7}	1.5×10^{-8}	ND
FDXC	5.6 ± 0.1	*	2.0×10^{-7}	2.0×10^{-9}
FDSKOV3	2.6 ± 0.3	*	1.0×10^{-7}	2.0×10^{-11}
FDCaco-2	2.3 ± 0.3	*	1.0×10^{-9}	2.0×10^{-11}
FDCaov3	2.2 ± 0.1	*	*	ND
FDOVCAR3	0.8 ± 0.1	*	*	ND

* $IC_{50} > 3 \times 10^{-7}$
ND = not determined

[a] Cells were treated with FA–ribonuclease–I^{125} for 1 hour, washed, counted in a gamma counter and assayed for total cell protein. The values shown represent the average number of picomoles of FA–ribonuclease–I^{125} bound per milligram of cell protein.

[b] FA–toxin conjugate was prepared using the EDC method to yield a conjugate containing 1–4 folates/toxin molecule.

[c] No FA was attached to this toxin.

within the endosomal network.[60] When the toxicities of the Cys^{287} thioether– and Cys^{287} disulfide–linked folate conjugates towards HeLa cells were compared, the former exhibited an IC_{50} of $>10^{-7}$ M, while the latter displayed a value of $2–4 \times 10^{-11}$M.[46] Clearly, the difference in potency of more than four orders of magnitude emphasizes the importance of incorporating some mechanism for transit from the endosome in the design of any folate targeted protein conjugate.

The value of a toxin translocation domain is heightened when the number of folate receptors on the targeted cancer cells is limited. As seen in Table 1, cells expressing large numbers of FRs (KB, HeLa and 2008-FBP cell lines) can be readily killed by both FA–momordin and FA–LysPE38. As anticipated, FA–LysPE38 was approximately 10-fold more toxic than FA–momordin in all cases. Cell lines FDXC, SKOV3, and Caco-2, which express a lower number of FR, are still susceptible to FA–LysPE38 and ricin (a toxin with activity similar to momordin, but with translocation capability); however, they are insensitive to FA–momordin. When co-cultures of Caco-2 cells (FR^+) and Hs67 cells (FR^-) are treated with FA toxins, FA–momordin does not retard the growth of the tumor cells, whereas Cys^{287} disulfide-FA selectively kills the Caco-2 cells without harming the Hs67 cells. This again suggests that FA–momordin does not reach

TABLE 2
Cytotoxicities of FA–Toxin Conjugates towards KB Cells

FA–toxin conjugate[a]	IC_{50}[b]
FA–momordin	1.0×10^{-9}
FA–PE38	1.0×10^{-10}
FA–PE35 (-S-C-)	$>10^{-7}$
FA–PE35 (-S-S-)	2.0×10^{-11}
FA–maize toxin	1.3×10^{-12}
FA–diphtheria toxin	1.0×10^{-11}

[a] FA-PE35 (-S-C-) stands for the PE35 conjugate linked to FA via a nonreducible thioether bond, while FA-PE35(-S-S-) refers to the conjugate bridged by a reducible disulfide bond. All other conjugates were prepared by the EDC method.

[b] After a 20 min exposure to FA–toxin conjugate, KB cell monolayers were washed with fresh growth medium and allowed to proliferate in the incubator for 47 h, prior to assay for protein synthesis activity.

the cytosol in lethal quantities, while toxins with a translocation domain do. Therefore, it can be concluded that below a threshold level of FR expression, only toxins with translocation capabilities can exert their cytotoxicities. It should be noted, however, that CaOV3 and OVCAR3 cells, which also express low levels of the FR receptor, are resistant to FA–LysPE38. This indicates that factors other than receptor density and translocation capability may also be important for FA–toxin cytotoxicity.[46]

A table summarizing the potencies of several folate–toxin conjugates towards cultured KB cells is provided for purposes of comparison (Table 2). The fact that both the unconjugated toxins as well as the FA–toxin conjugates in the presence of 1 mM free FA exhibit some residual toxicity suggests that small amounts of toxins can enter cells via nonfacilitated routes. However, the increase in potency of $>10^4$ upon derivatization also emphasizes the merit of exploiting the folate-uptake route for efficient intracellular delivery of the toxins. When the added merit of tumor-specific targeting is considered, folate would seem to constitute a ligand worthy of consideration for applications in chemotherapy.

VI. FOLATE-CHEMOTHERAPEUTIC AGENT CONJUGATES

While protein toxins may have the advantage of high molar toxicities, they simultaneously suffer from problems of reduced penetration into tumor masses and possible immunogenicity. Since these latter limitations are not shared by low-molecular-weight complexes of folic acid, folate conjugates of classical organic chemotherapeu-

tic agents would seem to constitute attractive alternatives to protein–toxin conjugates. For this strategy to succeed, however, four new obstacles must be overcome. First, although many cancer cells express $>10^6$ FR per cell, this receptor number is still insufficient to deliver toxic concentrations of several less potent chemotherapeutic agents unless each receptor recycles multiple times. The solutions to this problem are obvious, but not necessarily trivial to accomplish: one must either select a chemotherapeutic agent that is toxic at the levels achieved during one endocytic transit of the FR, or one must maintain elevated plasma levels of the folate conjugate for several hours in order to allow each FR to deliver multiple conjugate molecules. As will be noted below, with reasonable levels of plasma–folate conjugates maintained for 4 h, it is possible to deliver ~70 million conjugates into cancer cells *in vivo* expressing fewer than one million receptors per cell.[30] A second potential obstacle arises from hydrophobicity of both FA and most of the popular chemotherapeutic agents to which FA might be attached. Thus, if the conjugate of the two moieties is also hydrophobic, it may tend to interact nonspecifically with all cells, losing the high tumor-specificity characteristic of water-soluble folate conjugates. To be sure, screening protocols for effective chemotherapeutic agents have generally selected for drugs that can permeate cell membranes without the assistance of a facilitated uptake pathway. Furthermore, drug candidates that survive such screens are almost invariably lipophilic; they therefore bind cell membranes nonspecifically. As mentioned above, the ability to randomly permeate membranes or hydrophobically bind to lipid surfaces severely compromises the inherent tumor specificity of folate conjugates. Indeed, the drugs frequently discarded in classical cell cytotoxicity screens may in many cases constitute the ideal folate-targeted chemotherapeutic candidates, at least if the lack of toxicity towards cancer cells stems from their inability to enter the cells. Recent experience has in fact suggested that most popular chemotherapeutic agents can be converted to their folate-targetable forms by incorporation of water-soluble bridging groups between the folic acid and the drug. In this structural modification, the chemotherapeutic agents become nontoxic to cells that lack folate receptors, but highly lethal to cancer cells.

A third possible impediment to the development of low-molecular-weight drugs as folate-targeted chemotherapeutic agents lies in the tendency of small folate-conjugates to accumulate in the kidneys as well as in the tumor, at least in the mouse model (see below). However, even if this tendency holds true for the human kidney, it must be remembered that kidney retention of folate-conjugates is generally no worse than kidney retention of their nontargeted chemotherapeutic counterparts (which simultaneously accumulate in most other tissues at similar levels). Nevertheless, it would still seem prudent to explore methods of reducing folate-conjugate uptake by the kidneys in case nephrotoxicity emerges as a limiting factor in the use of low-molecular-weight folate conjugates. A list of possible strategies to avoid or reduce kidney uptake is provided in Table 3. Unfortunately, none of these have been tested yet *in vivo*.

Finally, one last obstacle to the use of low-molecular-weight folate–drug conjugates for cancer chemotherapy derives from the fact that some chemotherapeutic

TABLE 3
Methods for Reducing Folate Conjugate Uptake by Kidneys

- Lower urine pH with NH_4Cl and/or design a folate conjugate with a higher pKa of receptor dissociation
- Compete γ-linked folate conjugate with α-isomer conjugate or other folate analogs that bind to kidney but not tumor FRs
- Temporarily inhibit receptor recycling in the kidneys to prevent each FR from transporting multiple folate conjugates
- Chase treatment of folate–drug conjugate with high dose of free folate
- Increase size of folate conjugate to > 50,000 Da to prevent filtration by the kidneys
- Design a conjugate that is hydrolyzed at the kidney brush border membrane or during transcytosis from urine to blood and flush with water plus an osmotic agent such as mannitol
- Flush kidneys with a protective agent that prevents nephrotoxicity
- Employ a coupled drug that is nontoxic to kidneys

agents (e.g., doxorubicin) lose their toxicities when conjugated to other molecules such as folic acid. To circumvent this problem, linkages must be designed that are both sufficiently stable to survive the 10 to 60 min exposure to blood/interstitial fluids prior to folate receptor-mediated endocytosis, yet sufficiently labile to allow release of the active drug following uptake by the target cell. A number of esters, hydrazones, Schiff bases, and disulfide bonds potentially meet this requirement, with the disulfide linkage being most attractive, since reduction to the free thiols will in most cases only occur after entry into the target cell. Table 4 summarizes the properties desired in a chemotherapeutic agent to be used in the development of a tumor-selective folate–drug conjugate.

Several low-molecular-weight folate–toxin conjugates have been recently synthesized, but one is particularly noteworthy, since it overcomes most of the obstacles identified above. Ladino et al.[44] have linked FA to a maytansinoid, termed DM1, via a disulfide bond. DM1 blocks cell division and other essential cellular processes by disrupting microtubule formation.[44] The authors observe that FA-DM1 kills a panel of FR-expressing cancer cells (KB, SKOV3, LoVo, HeLa, and SW620), with IC_{50} values ranging from 10^{-11} M to 10^{-10} M. In contrast, the same drug conjugate is found to be nontoxic to cells lacking FR. This remarkable specificity is further shown to be due to the folate receptor, since folate-conjugate uptake is blocked by both excess free FA as well as antibody to FR. Clearly, with proper selection of the cytotoxic drug, fo-

TABLE 4
Desired Properties in Chemotherapeutic Agents to be Used as Folate-Targeted Conjugates

- Water solubility and membrane impermeability
- Low molecular weight
- High toxicity ($IC_{50} < 10^{-8}$ M)
- Modifiable functionality ($-NH_2$, $-OH$, $-C(O)R$, $-SH$)
- Active in conjugated form (or derivatizable with a cleavable linkage)

late-targeted chemotherapy shows considerable promise for development of tumor-specific antineoplastic agents.

VII. FOLATE-TARGETED RADIOIMAGING AGENTS

One of the most important practical problems in oncology today relates to inadequacies in the diagnosis of the grade and stage of a patient's tumor. As a result, many cancer patients receive either insufficient or excessive treatment, often with the wrong therapeutic agent. Furthermore, more than half of patients with cancer are found to have metastatic disease at the time of first detection.[61] Three-dimensional imaging methods, such as high-resolution computed tomography (CT) and magnetic resonance imaging (MRI), are excellent for viewing normal tissues and defining the anatomy in the region of a tumor, but these methods are expensive and often fall short in detecting malignancies at an early stage. In contrast, while radiological detection methods may lack the resolution of CT and MRI, if the radio signal is emitted only from the tumor mass, the method may allow detection of remote metastatic disease before it can spread further. Thus, when the radionuclide is connected in some manner to a tumor-specific ligand such as an antibody, peptide hormone, or folic acid, it can concentrate in the tumor and reveal its location, distribution, and size.

Gallium-67, a γ-emitting radionuclide, has been used for detection of certain tumors.[62] Administered in the form of an aqueous citrate complex, ^{67}Ga is rapidly taken up by transferrin, a plasma protein, and delivered to cells (e.g., cancer cells) overexpressing the transferrin receptor.[63,64] The clinical use of ^{67}Ga as an imaging agent is restricted, however, due to its slow clearance from nontarget tissues and poor tumor/background ratios. Fortunately, deferoxamine (DF), a trihydroxamate ligand, forms a stable octahedral coordination complex with trivalent cations like $^{67}Ga^{3+}$ and thereby prevents ligand exchange with plasma transferrin. Deferoxamine also contains a free amine that is separated from the hydroxamate group by five methylene units; this amine

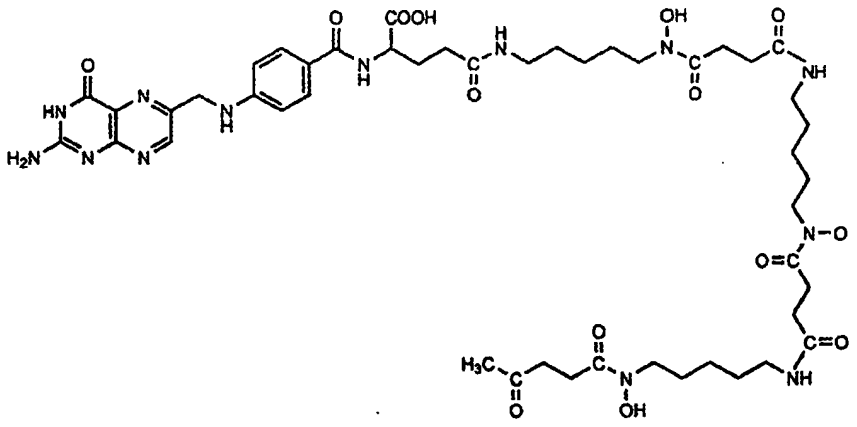

FIGURE 5. Structure of FA–deferoxamine conjugate.

can be used for covalent attachment of DF to targeting agents like antibodies without compromising chelating properties.[65–67] (See Figure 5.) To evaluate the potential of a radiolabeled FA conjugate in tumor diagnostic imaging, FA was covalently linked to DF via an amide bond using the DCC method,[54] and the γ-isomer of FA–DF was separated from the α-isomer by anion-exchange chromatography. Both γ- and α-FA–DF conjugates were radiolabeled with $^{67}Ga^{3+}$ and tested for uptake by cultured KB cells. Competitive binding studies revealed that only the γ-FA–DF was recognized by the FR on KB cells, interacting with an affinity comparable to unconjugated FA. Kinetic studies further revealed that the uptake was very rapid, with half maximal (4.8×10^6 molecules) cell association in approximately 3 min. The cellular uptake of γ-FA–DF–^{67}Ga was more than 100 times as efficient as the uptake of ^{67}Ga-citrate, confirming the importance of receptor involvement in the internalization mechanism.

The ability of γ-FA–DF–^{67}Ga to target tumor cells *in vivo* was examined using an athymic mouse tumor model. Subcutaneous inoculation of 4×10^6 KB cells into athymic mice yielded approximately 0.2 g tumors in 15 days, at which time either γ-FA–DF–^{67}Ga, DF–^{67}Ga, or ^{67}Ga-citrate was administered by intravenous injection. Since normal rodent chow contains a high concentration of FA (6 mg/kg chow), some mice were placed on an FA-free diet for 3 weeks prior to treatment to achieve serum folate concentrations of approximately 25 ± 7 nM, which is close to the range for normal human serum (15 ± 10 nM).[29] The γ-FA–DF–^{67}Ga conjugate showed a high tumor-specific deposition *in vivo*, with 5% ± 2% of the injected dose/g tumor (n = 3 mice, ± S. D.) at 4 hr postinjection. The corresponding tumor-to-background ratios at 4 h postinjection

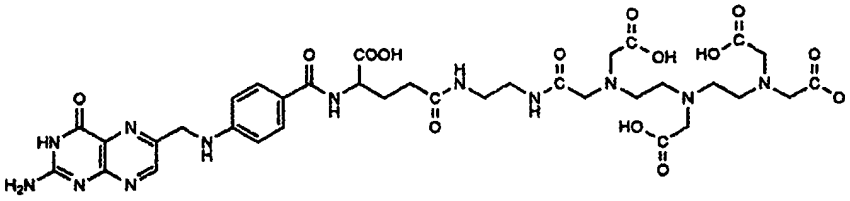

FIGURE 6. Structure of FA–DTPA.

were: tumor/blood, 400 ± 200; tumor/muscle, 120 ± 50; tumor/liver, 11 ± 3; and tumor/kidney, 2.6 ± 0.9.[29] The tumor uptake of γ-FA–DF–^{67}Ga conjugate was dramatically reduced by co-injection of 2.4 ± 1.0 mg FA or by maintaining the animals on normal FA-rich rodent chow. In control experiments, ^{67}Ga-citrate exhibited tumor uptake of 10.9% ± 0.2% of the injected dose/g tumor (n = 3 mice, ± S. D.), but very poor target-to-background contrast (tumor/blood was 0.84 ± 0.19, tumor/muscle was 5.4 ± 0.7, tumor/liver was 2.3 ± 0.2, and tumor/kidney was 2.4 ± 0.3). Unconjugated ^{67}Ga–deferoxamine showed no tumor affinity. The high tumor/background contrast of γ-FA–DF–^{67}Ga is probably due to its high specificity and small size, which allows for rapid clearance from the blood and other nontargeted tissues. However, approximately 30% of the administered γ-FA–DF–^{67}Ga was excreted through the hepatobiliary route into the intestines. This slow clearance rate through the GI tract unfortunately interfered with rapid visualization of abdominal tumors, suggesting a need for an improved imaging agent that would quickly clear through the kidneys.

Because of its high water solubility, diethylenetriaminepentaacetic acid (DTPA) was selected for use as the next generation folate-targeted imaging agent.[47] (See Figure 6.) The desired γ-FA–DTPA regioisomer was prepared by first conjugating ethylenediamine (EDA) to the γ-carboxyl group of FA by the methyl-folate method and further reacting the purified FA–EDA with DTPA anhydride. Chelation of ^{111}In, a γ-emitting radionuclide with a 67.4 h halflife, then completed assembly of the imaging agent. In KB cells, the kinetics of uptake of γ-FA–DTPA–^{111}In was found to be similar to that of free FA and γ-FA–DF–^{67}Ga.[47] The intravenously administered γ-FA–DTPA–^{111}In was found to concentrate in the KB tumors at a level of 3.1% ± 0.6% of the injected dose/g (n = 4 mice, ± S. D.) at 4 hours postinjection. High tumor/nontumor ratios were also observed at this time point, where the tumor/blood ratio was

340 ± 100, tumor/liver was 30 ± 10, tumor/muscle was 30 ± 10, and tumor/kidney was 1 ± 0.3.[30] The tumor/intestine contrast with γ-FA-DTPA-^{111}In was excellent, since only 3% of the agent was found to clear through the intestines, allowing rapid imaging of abdominal cancers. Mice that received γ-FA-DTPA-^{111}In with a simultaneous blocking dose of FA showed a 10-fold reduction of tumor radiotracer intensity. In contrast, mice that received a "chase" dose of FA 3 hours after radiotracer injection displayed only slightly diminished uptake. This suggests that most of the tumor-associated ^{111}In-tracer is internalized by the tumor cells within the above 3 h time span, and is therefore no longer available for competitive displacement by free folic acid. It was also shown that unligated ^{111}In-DTPA displayed no tumor affinity, while ^{111}In-citrate exhibited weak tumor uptake but poor tumor/background tissue contrast. When γ-FA-DTPA-^{111}In was diluted with varying doses of FA, the tumor uptake of γ-FA-DTPA-^{111}In at 4 h varied in a dose-dependent manner. At high radiotracer doses, tumor-cell uptake reached values of ~70 million molecules/cell. Since these KB tumors express < 10^6 folate receptors/cell, it can be concluded that these receptors must recycle many times during an average 4 h uptake period. Pharmacokinetic studies of γ-FA-DTPA-^{111}In biodistribution showed that tumor uptake of radiotracer increases rapidly during the initial 30 minutes but remains stable from 30 minutes to 24 hours postinjection. In γ-scintigraphy imaging studies, a KB cell tumors were readily visualized by 1 h following intravenous administration of γ-FA-DTPA-^{111}In and highly resolved by 4 h postinjection. By 24 h postinjection, unbound γ-FA-DTPA-^{111}In and γ-FA-DF-^{67}Ga were fully cleared from the body, with only tumor and kidneys displaying measurable levels of radioactivity. Microautoradiographic studies of kidney slices showed that the kidney-associated radio tracer was selectively localized in the proximal tubules. The tracer in the tumor, however, was evenly distributed among all cancer cells in the tumor mass.

Thus, FA-based radiopharmaceuticals were found to afford high tumor to nontarget tissue contrast for all tissues except the kidney, where the tracer uptake and retention roughly equaled that observed with the tumors. Quantitative evaluations revealed high levels of tumor cell uptake, possibly due to the ability of FR to recycle and facilitate multiple deliveries.

Challenges that still remain may involve reduction of kidney uptake (if this problem also occurs in humans) and the design and synthesis of ^{99m}Tc-based radioimaging agents. It may also prove beneficial to use the same technology to develop radiological agents for cancer therapy, as well as contrast agents (e.g., γ-FA-DTPA-Gd) for MRI.

VIII. STUDIES WITH FA-LABELED LIPOSOMES

Liposomes are unilamellar or multilamellar lipid assemblies that allow incorporation

of large quantities of hydrophilic molecules within their aqueous interiors and hydrophobic drugs inside the hydrocarbon regions of their bilayers.[68-70] Advantages of liposomal drug delivery include the ability to (i) protect the encapsulated drugs from degradation by destructive enzymes, (ii) prolong the circulation times of drugs that would otherwise be rapidly filtered by the kidneys,[71] (iii) promote more gradual release of drugs over time,[72] and iv) prevent immune recognition of normally immunogenic drugs. In the case of monoclonal antibody-derivatized liposomes, the encapsulated microcarrier systems allow the specific targeting of drugs in a concentrated manner to a desired tissue.[73-76] Previous problems associated with liposome instability and removal by the reticuloendothelial system have recently been diminished by optimizing liposome size, forming liposomes from saturated lipids and cholesterol, and incorporating surface protective agents such as gangliosides or polyoxyethylene-derivatized lipids to prevent unwanted binding of serum proteins and phagocytosis.[72,76-79] Liposomes constructed in this manner now survive up to 24 h in circulation compared with only 2 h for their unmodified predecessors.[77]

Since proteins, drugs, and imaging agents were shown previously to be delivered into folate receptor-bearing cells by folate derivatization, liposomes conjugated to FA were also tested for targeting to neoplastic tissues.[80] Unfortunately, polyethylene glycol coating (previously shown to be important for liposome survival *in vivo*) was immediately found to prevent receptor recognition when the folate was directly linked to a phospholipid head group. To circumvent this problem, FA was attached to the distal ends of a few lipid-conjugated PEG molecules, allowing the targeting ligand to reach receptor sites far from the liposome surface (Figure 7). These liposomes were found to efficiently target FR-bearing tumor cells with as few as 0.2% of the bilayer lipids derivatized with folate (Figure 8). More intensive folate labeling did not significantly enhance cellular uptake, but spacer length was found to be important, since internalization of folate-targeted liposomes constructed with a 250 Å PEG spacer was ~37-fold more efficient than uptake of similar liposomes with a ~23 Å spacer.[80] Time course studies of FA–PEG-liposome endocytosis by KB cells also revealed that cell association increased linearly over the first hour and gradually saturated by four hours (unlike uptake of FA–protein conjugates, which saturated within 30 minutes). While it can be suggested that the difference in kinetics between proteins and liposomes might be due to the slower penetration of the larger liposomes through the carbohydrate layer on the cell surface, further studies will have to be conducted before this conjecture is confirmed.

Analysis of the absolute number of FA–PEG-liposomes taken up by folate receptor-bearing cells shows that only 2.5×10^5 liposomes typically associate with KB cells at saturation in comparison to ~ 4×10^6 FA–protein conjugates. We assume that the binding of an FA–PEG-liposome to one receptor on a cell surface can sterically block binding of other liposomes to adjacent receptors. Alternatively, since each liposome may contain up to several hundred folate tethered lipids, it is not unlikely that each liposome might occupy multiple receptors in a localized receptor cluster. In support of this latter hypothesis is the unusually high affinity exhibited by FA–PEG-liposomes

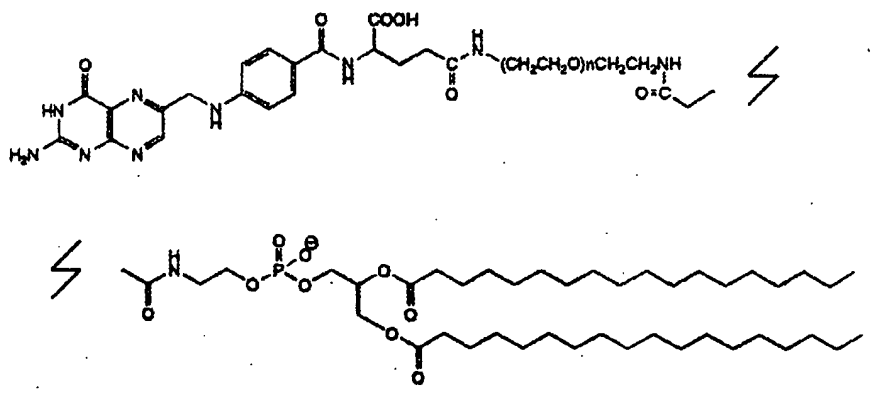

FIGURE 7. Structure of FA–PEG–DSPE. The polyethylene glycol bridging group used in most studies [PEG, –NH(CH$_2$CH$_2$O)nCH$_2$CH$_2$NH–] had an average length of 250 Å and M.W.~3350. (The squiggle denotes where the molecule continues on the second line.)

for folate receptor–expressing cells. Thus, when a multivalent ligand docks simultaneously with multiple cell-surface receptors, the final affinity is proportional to the product, not the sum, of all individual binding constants. Consequently, competitive inhibition of FA–PEG-liposome binding to folate receptor–bearing cells is impossible at submillimolar concentrations of FA.

Although FA–PEG-liposomes are seen to rapidly enter FR-expressing cells, one obstacle that can limit the potential application of liposomes as drug-delivery vehicles is their low unloading efficiency following receptor-mediated endocytosis.[70] This problem has been further aggravated by the requirement to construct highly stable liposomes that can survive the rigors of circulation. Unfortunately, the enhanced stability required for prolonged survival in the blood stream can simultaneously inhibit liposome unloading following cell uptake. To circumvent this obstacle, two primary strategies have been pursued. First, fusogenic peptides—such as the amphipathic peptide with the repeat unit of glutamic acid–alanine–leucine–alanine (EALA)—that acquire their fusogenic properties only after exposure to low endosomal pHs have been employed to promote selective unloading of internalized liposomes. In the case of EALA, the repeating unit places glutamic acid residues on the same face of the peptide's α-helix in such close proximity that they repel each other when ionized (pH > 6.0), promoting peptide unfolding. At endosomal pHs (pH ~ 5), however, the glutamic acids are protonated, allowing the peptide to coil into an amphipathic helix that displays high affinity for lipid bilayers. Insertion of EALA at endosomal pHs destabilizes the bilayer, promoting its fusion with adjacent membranes. The EALA peptide was modeled after the GALA peptide[81] but with additional helix forming amino acids, resulting in a peptide that achieved its maximal helical content at pH 6 instead of pH 5.[82] Since endosomal pH gradually decreases as the endocytosed ligand traf-

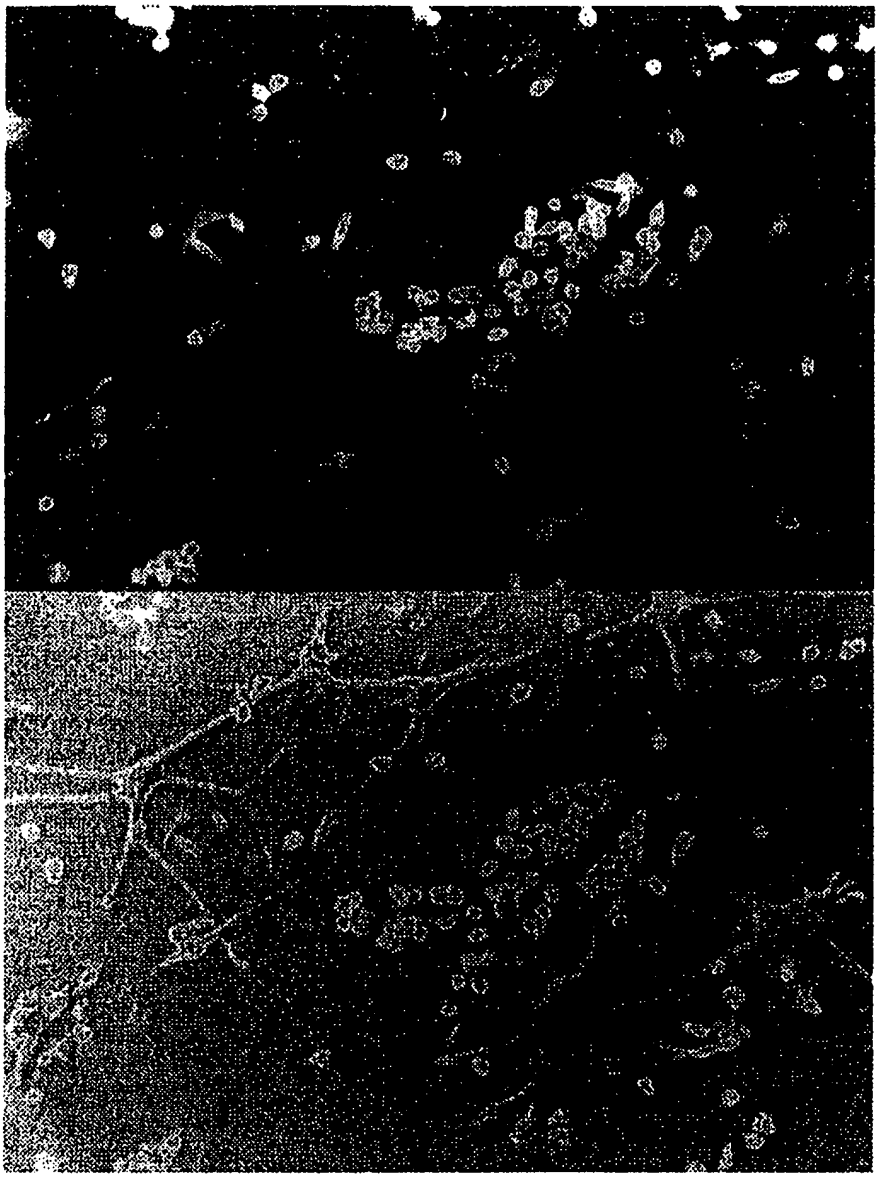

FIGURE 8. Selective uptake of FA–PEG-liposomal calcein by tumor cells (HeLa cells) in HeLa/WI38 co-cultures. HeLa/WI38 co-cultures were treated with FA–PEG-liposomal calcein and photographed in both the fluorescence (dark fields) and phase-contrast mode (bright fields) on a microscope. HeLa cells appear as small round or polygonal cells, while the WI38 cells are considerably larger with a dendritic or fibroblast-like morphology. (Photo courtesy of Dr. Robert J. Lee.)

fics further into the cell,[83,84] the higher coil-to-helix transition pH of the EALA peptide is thought to enable release of the liposomal contents prior to deposition into lysosomes. Although the fusogenic peptide can either be entrapped free within the FA–PEG-liposome or covalently ligated to the liposome's surface, the former protocol is preferred, since it should not induce a significant immune response against the fusogenic peptide.

The alternative strategy for promoting FA–PEG-liposome unloading following endocytosis is to include in the liposome formulation a lipid that acquires fusogenic properties only after entry into target cells. Although many attempts to design and synthesize such "highly trained" lipids have been reported, virtually all of the derived liposomes have failed to properly function when tested in the presence of serum. Recently, however, we have developed a "caged cationic lipid" that actually displays improved drug-delivery properties in the presence of serum (unpublished data, see below). It will now be important to identify the optimal formulation of this novel lipid with other lipids for specific applications in drug and gene delivery.

A. Doxorubicin Liposomes

Doxorubucin (adriamycin, abbreviated hereafter as Dox) is a potent anticancer agent with activity against a variety of carcinomas. Its use is limited by toxic side effects, such as dose-related cardiomyopathy. To circumvent this cardiotoxicity, liposomal doxorubucin formulations prepared by a "remote loading" technique (whereby the drug is loaded with high efficiency into liposomes in response to a pH gradient) have been developed.[85] A similar technique has been used to prepare various doxorubicin containing FA–PEG-liposome formulations. These latter liposomes are ~130 nm in diameter with roughly 75 folate ligands on the outer surface of each liposome. Because cellular uptake of doxorubicin can be readily monitored spectrophotometrically, a comparison of drug uptake by KB and HeLa cells was made for folate-targeted liposomes (Dox), nontargeted liposomes (Dox), and free doxorubicin.[55] KB cell-associated doxorubicin fluorescence due to uptake of FA–PEG-liposomes (Dox) was found to be 45 and 1.6 times higher than nontargeted liposomes (Dox) and free doxorubicin, respectively. Importantly, uptake of the folate-targeted drug (1.8×10^{10} molecules/cell) by KB cells was reduced ~ 70% by pretreatment with 1 mM FA, indicating that partial competition is possible at high free folate concentration. It was further shown that addition of 4-mol% PEG2000-DSPE in the formulation in order to prolong *in vivo* circulation did not reduce the uptake of FA–PEG-liposomes (Dox). Thus, the 250 Å PEG spacer between FA and the surface of the liposome was sufficient to overcome the steric hindrance caused by the forest of PEG molecules on the liposome surface. Comparison of the cytotoxicities of various doxorubicin formulations showed that the IC_{50} value of FA–PEG-liposomes (Dox) was 86 times lower than nontargeted liposomes (Dox), but only 2.7 times lower than free doxorubicin. Although FA–PEG-

liposomes (Dox) were only marginally more potent than free doxorubicin, free doxorubicin is cleared 450 times faster from systemic circulation than PEG-liposomes (Dox),[79] and free doxorubicin is also considerably more toxic to the heart. Consequently, FA–PEG-liposomes (Dox) would seem to constitute an attractive alternative to free doxorubicin for treatment of tumors expressing FR, especially if pH-sensitive liposomes can be employed to promote intracellular unloading of the drug.

In view of the previously described ability of folic acid to target covalently linked low-molecular-weight drugs and imaging agents to tumors with high efficiency and high selectivity, the question naturally arises whether a clinical niche can ever be developed for folate-targeted liposomes in cancer chemotherapy. Although the small (but rapidly growing) data base is still insufficient to allow firm conclusions to be drawn, the merits and deficiencies of both direct folate ligation and liposome entrapment are becoming clearer, and they can be presented here for comparison. The advantages of FA–PEG-liposome–mediated drug delivery are (i) the therapeutic drug need not be derivatized, but can be simply entrapped in unmodified form in a folate-targeted liposome, (ii) the circulation halflife of the drug can be significantly extended by liposome encapsulation, (iii) a larger number of drug molecules can be delivered at each receptor/receptor cluster, (iv) potentially immunogenic drugs can be hidden from the immune system until internalized by FR-expressing cells, (v) hydrolytically sensitive drugs such as proteins and nucleic acids can be protected from digestive enzymes in the plasma and extracellular milieu during delivery, and (vi) specific folate receptor–mediated uptake by the kidneys can be prevented due to the inability of the liposomes to filter across the glomeruli.

The disadvantages of the folate-targeted liposome strategy stem almost entirely from two sources. First, because of their large sizes (often near 100 nm in diameter), liposomes perfuse most tumors very poorly, bathing mainly those cells near capillary walls while rarely penetrating the more distant regions of the cancer mass. The liposome's large size and propensity for binding serum proteins also renders it an easy target for phagocytosis by macrophages of the reticuloendothelial system. Low-molecular-weight folate conjugates, in contrast, appear to access virtually all cells in a tumor (see data on imaging agents) and largely escape uptake by macrophages. Second, since the targeting ligand (folic acid) is attached to the liposome and not the drug directly, any leakage or rupture of the lipid bilayer releases untargeted drug into circulation. Except where this release is concentrated near the tumor mass, all tumor specificity of the leaked drug is lost.

Direct ligation of folic acid to low-molecular-weight drug molecules, as noted above, is also characterized by its own set of strengths and weaknesses. Prominent among the strengths are (i) the ability of the folate-linked drug to access most cells in both primary and metastatic cancer tissue regardless of tumor size or location, (ii) the ability of the folate–drug conjugate to avoid removal by macrophages, (iii) the capacity of the folate–drug complex to release from its folate receptor following trafficking into acidic endosomal compartments, thereby allowing the same receptor to recycle to the cell surface and carry multiple folate conjugates into the cell (folate–PEG-liposomes

generally form multiple attachments with adjacent folate receptors and are therefore not easily dislodged at low endosomal pH), and (iv) the very low level of uptake by nontumor cells except in the kidney (although the mouse kidney appears to express FR at a level comparable to human cancers, quantitative information is currently lacking on the level of FR expression in the human kidney).

The primary weakness of the direct ligation strategy is that many chemotherapeutic agents become inactive or less active when conjugated to carriers or targeting ligands, and therefore they require attachment via a cleavable linkage. Also, as elaborated above, direct folate-drug conjugates must in certain cases be modified to reduce their hydrophobicities (nonspecific binding), and a prolonged delivery protocol may have to be implemented if sustained serum levels are required for efficacy. Finally, the smaller conjugates are filtered more readily by the kidneys and thereby may obtain easier access to the apically oriented FR in the proximal tubules. Although each of the above shortcomings can be alleviated, it would still seem prudent to evaluate each drug independently to identify the optimal method for its selective delivery to tumor cells.

IX. FOLATE-TARGETED NON-VIRAL GENE DELIVERY

There are clearly a number of obstacles limiting successful gene therapy, but the most difficult to overcome has been the inability to transfer a sufficient quantity of the desired gene to achieve a measurable therapeutic effect. Below we will summarize the two major strategies currently employed in gene therapy (use of viral and nonviral vectors) and describe how folate-mediated targeting has contributed to the development of each strategy.

Compared to viral vectors, synthetic lipid-based gene-delivery systems have several distinct advantages. First, DNA/lipid complexes are generally easier to prepare in bulk quantities with the purity required for human use. Second, there are fewer limitations on the sizes of genes that can be transferred using liposomal-delivery systems. Because most liposomal vectors lack proteins, they can also be expected to evoke a much lower immune response (if any). Finally, lipid-based vectors have a lower risk of recombining to generate an infectious form of the vector or of inducing tumorigenic mutations in the target cells. Thus, liposomal vectors have many attributes that would appear to justify their further development.

The most widely used liposomal DNA delivery systems are those comprised of cationic lipids. These positively charged amorphous aggregates avidly complex with negatively charged DNA and are taken into cells primarily through charge interactions.[86] Although such liposomal vectors are capable of transforming a broad range of cell types with good efficiency, they simultaneously suffer from the disadvantage of displaying little target-cell specificity and relatively high cytotoxicity. Consequently, receptor-targeted lipid formulations either with or without partial participation of cationic lipids have attracted considerable attention. The two targeting ligands that

have enjoyed the greatest success to date are transferrin and asialoglycoprotein—i.e., ligands that find their greatest application in gene therapy of hematopoietic/cancer cells and hepatocytes, respectively.[87-91] More recently, folic acid has also been used for selective transfection of cancer cells. These latter studies will be reviewed below.

Early work on FR-mediated gene therapy focussed on plasmid vectors compacted with a polylysine polymer that in turn was intermittently derivatized on its ε-amino groups with FA.[92,93] Curiously, when these FA-polylysine-DNA complexes were initially used to deliver a β-galactosidase gene into various tumor cells overexpressing FR, only low levels of β-galactosidase activity (< 0.005 units/ mg protein) were detectable, and usually less than 0.1% of the cells stained positive for β-galactosidase. In contrast, when a replication-defective adenovirus was co-incubated with the FA–polylysine–DNA complexes, a 1000-fold increase in β-galactosidase activity was observed, and 20% to 30% of the cells were found to stain positive.[93] Since the inactivated adenovirus is thought to act primarily as an endosome disrupting agent, this observation supports the earlier conclusions that an endosome escape mechanism must be supplied to enable full function of any FR-transported macromolecule. In fact, to further document that the adenovirus served primarily to facilitate endosome release rather than vector endocytosis, Gottschalk et al.[93] showed that β-galactosidase gene expression could be inhibited by addition of excess free folic acid even in the presence of adenovirus. The requirement for endosome unloading was further bolstered by the observation that chloroquine, a weak base that raises the pH in endosomes and thereby inhibits their routing to lysosomes, also enhanced β-galactosidase gene expression.[94] Indeed, as will be noted below, the major obstacle in folate-mediated gene therapy may lie more in improving endosome release following FR-mediated uptake than in enhancing the initial step of vector delivery into the cell.

Another vector in which DNA has been condensed with polylysine and targeted with FA was developed by Lee and Huang.[95] In this strategy, the compacted DNA was prepared with a slight excess of polylysine to yield a particle with a net positive charge. This cationic charge was then exploited to promote encapsulation of the condensed vector into a dispersion of anionic (cholesterol hemisuccinate) and neutral (dioleylphosphatidylethanolamine) lipids labeled with a small fraction of the modified lipid, folate-polyethyleneglycol-phosphatidylethanolamine (FA–PEG–PE), for tumor-specific targeting. Importantly, the charge interaction between the condensed vector and anionic lipid species enabled highly efficient loading of the plasmid DNA into the folate-targeted liposomes. The resulting liposomes were found to be spherical with a mean diameter of 70 nm ± 10 nm, containing a core of highly compacted DNA.

Transfection studies with the above vector revealed that the efficiency of gene expression was affected by both lipid-to-DNA ratio as well as lipid composition. At lipid-to-DNA ratios of 4 and 6, where the lipid particles had a net positive charge, high transfection levels were obtained, but gene expression was not blocked by free FA, suggesting that gene delivery was not measurably facilitated by folate receptor–mediated endocytosis. In contrast, at high lipid-to-DNA ratios (e.g., 10 to 12), where the net charge on the liposomes was negative, gene transfection was strongly

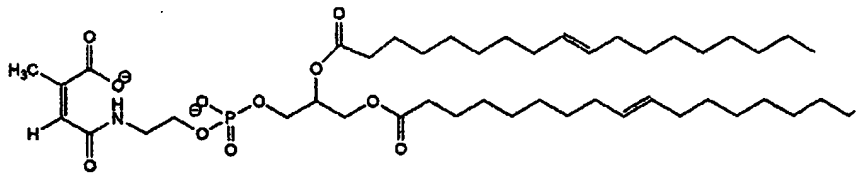

FIGURE 9. Structure of Citraconyl–DOPE.

inhibited by free FA. Indeed, transfection of CHO cells that lack folate receptors was readily mediated by the former formulation but not the latter, emphasizing that liposomes with a net negative charge can only enter cells efficiently by folate receptor-mediated endocytosis.

In addition to defining electrostatic charge requirements for folate-mediated gene delivery and exploring a novel approach for DNA encapsulation, the Lee and Huang study[95] also confirmed the requirement of an endosome escape mechanism for efficient gene expression. For most experiments, the authors employed a mixture of pH-sensitive fusogenic lipids, dioleylphosphatidylethanolamine and cholesterol hemisuccinate, in their lipid formulation to assure vector unloading following receptor-mediated endocytosis. In this composition, the dioleylphosphatidylethanolamine provides the driving force for fusion of the liposomes with endosomal membranes, while the cholesterol hemisuccinate supplies the pH-dependent trigger that prevents fusion until a low (endosomal) pH is encountered. Consistent with these functions, substitution of dioleylphosphatidylcholine (a nonfusogenic lipid) for the dioleylphosphatidylethanolamine was observed to abrogate gene expression. Thus, loss of DNA unloading led to loss of gene expression.

Recognizing the importance of an efficient endosome escape mechanism for successful gene transfer, Reddy and Low[96] have recently explored a different avenue for triggering release of encapsulated plasmid DNA from endosomes following receptor-mediated endocytosis. In this formulation, a polylysine condensed pCMVβ gal expression vector was entrapped in FA-targeted anionic liposomes similar to those developed by Lee and Huang, except the latter liposomes contained N-citraconyldioleylphosphatidylethanolamine[97] as the primary pH-sensitive lipid (Figure 9). This "caged" pH-sensitive lipid prefers to associate into stable phospholipid bilayers until it is hydrolyzed at endosomal pHs to yield the strongly fusogenic dioleylphosphatidylethanolamine. Thus, at endosomal pHs, the entire liposomal vector is transformed to a fusogenic state and the nucleic-acid contents are discharged through the fusion pore into the cell's cytoplasm. In a direct comparison with the Lee and Huang formulation, this caged fusogenic lipid was found to enhance the transfection efficiency of KB cells by a factor of 4 to 5, suggesting that improvements in folate-targeted gene therapy can still be made by development of novel endosomal escape mechanisms.

When the caged liposome complexes were administered to tumor cell lines expressing different levels of FR, transfection efficiencies were found to correlate directly with FR content, and gene expression could be inhibited by pretreatment of the cells with an excess of free folic acid. Taken together, these data demonstrate that FR-mediated endocytosis constitutes a viable route for targeted gene delivery, and that the next generation of liposomal vectors will likely contain significant improvements in strategies for endosomal release and transport of the vectors into the nucleus.

X. FOLATE-TARGETED VIRAL VECTORS

Human adenovirus contains a 36 kb double strand of DNA that encodes more than 50 gene products required for various events in the viral life cycle. By eliminating the E1 region of the viral genome, space is made for inserting therapeutic genes into the vector, and in the absence of the transactivating E1a protein, the virus cannot replicate. Thus, after gene transfer, no viral spread is expected to occur and transgene expression should be facilitated. In contrast to retroviruses, adenoviral vectors can be concentrated to high titer (10^{12}/mL), can transduce nondividing cells, and do not integrate into host chromosomal DNA.

The cellular receptor for adenovirus is widely expressed on the surfaces of mammalian cells, and therefore the virus is not intrinsically targeted to specific cell types.[98] Consequently, to permit gene delivery to selected cell types, it is necessary to block the endogenous viral tropism and introduce a novel targeting ligand. The binding of adenovirus to its yet unidentified cellular receptor is mediated through the knob domain of the fiber capsid protein.[99] Since the amino-acid residues of the knob responsible for binding have not yet been identified, site-directed mutagenesis is not currently an option for eliminating the virus' natural affinities. Therefore, Douglas et al.[52] elected to block the endogenous viral tropism by employing a neutralizing Fab fragment of an anti-knob monoclonal antibody (Mab). The neutralized virus was then retargeted to FR-expressing cells by conjugating the Fab to FA. Importantly, the FA redirected virus containing a luciferase reporter gene was found to enable adenoviral transfection of KB cells via the FR at a level comparable to that achieved by native unmodified virus. Without FA retargeting, the Fab-neutralized virus displayed only 1% of its normal transfection efficiency. FR-expressing KB cells could also be selectively transfected in the presence of FR-negative Jiyoye cells, confirming that the modified virus was dependent on FR for cell association. Curiously, although the virus appears to bind to the cell surface through the FR, it is then internalized via its native endocytotic pathway[100,101] mediated by the secondary interaction of the penton base with integrins on the cell surface.[102,103] Therefore, modification of the first step of adenoviral infection, the binding of the knob domain, does not apparently affect the ability of the virus to accomplish its subsequent entry steps of internalization and transport to the nucleus. Finally, evidence of this targeting strategy for cancer gene ther-

apy was provided by complexing an adenoviral vector carrying the gene for *herpes simplex* virus thymidine kinase (HSV-TK) with the FA–Fab conjugate to achieve specific killing of KB cells on treatment with ganciclovir.

Although the above study demonstrates that retargeting adenoviral vectors to FR-expressing cells might constitute a viable strategy for gene therapy of cancer, the data so far are very limited, and many questions still remain. For example, what measures must be taken to avoid elimination of the viral vector by an active immune system? Will other kinds of viral vectors be capable of escaping the endosomal compartments entered by folate conjugates? (Results to date indicate that at least two retroviral vectors cannot escape.) Can the natural tropism of a virus be mutated/suppressed without compromising the virus' ability to deliver its genome first into the cytoplasm and then on to the nucleus? Do some viral vectors exceed the maximum particle size that can be internalized by FR endocytosis? (We have observed that uptake of folate-targeted liposomes decreases sharply at diameters above 150 nm.) Clearly, much work remains before the full potential of FA-retargeted viral gene therapy can be accurately assessed.

XI. FOLATE-MEDIATED ANTISENSE THERAPY

Antisense therapy relies on the ability of short oligodeoxyribonucleotides (ODNs) to specifically hybridize with target mRNA transcripts through Watson–Crick base pairing. The formation of this ODN:RNA heteroduplex results in mRNA inactivation/degradation and the consequent inhibition of synthesis of the selected protein product.[104,105] A number of challenges in the preclinical development of antisense oligonucleotides have been identified, including ODN instability and poor uptake by target cells. The instability of natural ODNs has been largely overcome by using modified backbone chemistries, such as methylphosphonates and phosphorothioates.[106,107] Cationic liposomes and polylysine have also been investigated for their ability to enhance intracellular delivery[108,109]; however, most protocols still rely on a low-capacity natural ODN uptake pathway thought to be present in many cells. Clearly, for cancer applications, the development of a facilitated delivery methodology might greatly enhance the value of antisense therapy.

The epidermal growth factor receptor (EGFR) is overexpressed in a variety of human cancers[110,111] and is involved in transduction of cell growth signals.[112] EGFR antisense RNA has been shown to block expression of EGFR and thereby suppress the transforming phenotype of KB cells.[111] Antisense ODNs targeted to the EGFR were therefore encapsulated into egg phosphatidylcholine cholesterol/FA–PEG–PE liposomes (FA–PEG-liposomes)[113] and delivered into cultured KB cells. The ODNs used were all pentadecameric deoxynucleotides complementary to the EGFR stop codon; however, some samples were constructed with phosphodiester backbones while others contained three phosphorothioate linkages at each terminus. Cellular uptake of

the antisense encapsulated in the FA-PEG-liposomes was found to be nine times higher than uptake of antisense encapsulated in nontargeted liposomes and 16 times higher than uptake of unencapsulated antisense. When the FA-PEG-liposomal ODNs were labeled with fluorescein, ODN fluorescence could be observed by 24 h throughout the cytosol, suggesting that considerable escape of the antisense from the endosomes had occurred.

Brief treatment (4 h) of KB cells with the FA-targeted antisense constructs resulted in marked growth inhibition and morphological changes.[113] Although both folate-targeted phosphodiester and phosphorothioate ODNs strongly inhibited KB cell proliferation (>90%) and virtually eliminated EGFR expression, the same concentration of free (nontargeted) phosphodiester antisense exerted virtually no effect on cell proliferation and the same amount of free phosphorothioate ODN reduced growth by only 17%. Importantly, this difference in potencies between the two backbone chemistries was not observed for the respective ODNs delivered in folate targeted liposomes, probably because the protection offered by liposome encapsulation and facilitated internalization obviated the need for the more stable phosphorothioate backbone. However, following longer culture periods (> 48 h), maintenance of growth inhibition was more dramatic for the FA-targeted phosphorothioate ODN than for its natural counterpart, presumably because of the greater phosphorothioate stability in the cell cytoplasm. In neither case, however, was growth inhibition sustained or apoptosis induced. Rather, several days after the 4 h exposure to antisense, cell proliferation gradually returned to normal. Cell morphology—which, during the inhibited phase, had assumed a differentiated and shrunken, fibroblast-like appearance—also returned to its normal transformed state. Nevertheless, suppression of EGFR expression was profoundly evident for at least a 24 h period; had an apoptosis-inducing antisense construct been selected, considerable tumor cell killing might have ensued.

With the objective of improving endosome unloading, folate-derivatized liposomes comprised of fusogenic lipids have also been examined for their targeted antisense delivery.[114] As anticipated, antisense potency was measurably enhanced. It will be important in the future to develop targeted liposome preparations that can survive systemic circulation, yet still fuse with endosomal membranes following cell uptake.

Finally, not all folate-mediated antisense delivery protocols have relied on liposome encapsulation. In fact, in the case of the c-myb protooncogene, polylysine condensed-antisense ODNs were targeted to FR-expressing cells by direct FA derivatization of the polylysine.[53] Subsequent exposure of HL-60 cells to this antisense formulation resulted in significantly greater suppression of c-myb expression than was observed with free antisense,[53] even though HL-60 cells express only very low levels of FR (personal observations). Furthermore, inhibition of cell proliferation and induction of apoptosis were also observed. In a related example, a ribozyme construct that selectively cleaves the BCR/ABL oncogene was complexed with FA–polylysine and delivered into murine myeloblasts transformed with the bcr–abl gene (32D cells) by folate receptor–mediated endocytosis. The FA-targeted complex resulted in a 1,000-fold reduction in BCR/ABL mRNA when analyzed by reverse transcriptase–PCR.[115,116]

Apparently, protection of the targeted ODN within a liposome is not required for successful antisense/ribozyme therapy. Although direct ligation of FA to a backbone-stabilized antisense has never been reported in the literature, given the promising results seen in the above two macroscopic antisense complexes, an exploration of a direct attachment strategy should also be pursued.

XII. FOLATE-MEDIATED IMMUNOTHERAPY

Although the immune system is capable of attacking and eliminating tumors, it frequently fails to do so because tumor-specific antigens are not presented in the correct context or in sufficient quantities for immune recognition.[117] To efficiently mediate *in vivo* cytotoxicity, effector cells such as macrophages, natural killer cells, or cytotoxic T cells must be attracted to and activated within the tumor. One way of directing the immune response towards a tumor cell is to introduce a bispecific antibody that simultaneously recognizes a unique antigen on the tumor cell and a classical marker on a freely migrating immune cell. When the migration path of the immune cell takes it near the tumor cell, antibody-mediated crosslinking will result, greatly enhancing the chances of cancer-cell recognition.[118]

Kranz et al.[51] have reported the preparation of an innovative set of bispecific antibodies that consists of FA covalently linked to anti-T-cell receptor (TCR) antibodies. The three different anti-TCR antibodies used were a clonotypic antibody specific for the cytotoxic T cell (CTL) clone 2C; an anti-Vβ antibody, which recognizes TCR epitopes in the Vβ region; and an anti-CD3 antibody. The FA–antibody conjugates containing an average of five FAs per molecule exhibited high affinity (50 nM to 90 nM) for FR-expressing tumor cells but did not bind to FR-deficient cells. To test the targeting efficiency and specificity of the FA antibodies, five different mouse tumor cell lines that expressed either the α- or β-isoform of FR were examined using a ^{51}Cr-release assay to quantitate cell lysis. T cell–mediated lysis of FR$^+$ cell lines could be detected at FA–antibody concentrations as low as 1 pM, which was 1/1,000th the concentration required to detect cell binding to the FR$^+$ cells. Furthermore, the extent of lysis was found to correlate with the level of cell surface FR and was completely inhibited by free FA. Since normal cells express either no FR or greatly reduced levels of receptor, a window of conjugate concentration was envisioned where normal cells would escape immune targeting while cancer cells would be eliminated.

Unfortunately, a major problem that remained with the above approach was that the large size of the anti-T cell antibodies (Mr ~160,000) greatly restricted their access to poorly perfused regions of solid tumors. As anticipated, biodistribution studies with IgG, Fab2, Fab and single-chain variable region antibodies (scFv) revealed that scFv molecules (Mr ~30,000) penetrated tumors more rapidly, to a greater depth, and more uniformly than all other forms of the antibody. Therefore, the Kranz group[119] elected to construct an FA–scFv conjugate directed against the T-cell receptor. In this

fusion protein, the Vl and Vh regions of the scFv were linked by a 26 amino-acid flexible spacer that contained 8 lysine residues accessible for FA coupling. Importantly, the resulting FA–scFv conjugates were found to be as effective as FA–antibody conjugates in mediating lysis of tumor cells by CTL *in vitro*. Live animal studies still remain to be conducted with this novel construct.

Using a related approach, Canevari et al.[120] joined one chain from a monoclonal antibody against the human FR to a second chain from an anti-CD3 (T cell) antibody to promote T-cell recognition and lysis of tumor cells. The resulting bispecific antibody was found to be highly toxic to ovarian cancer cells when incubated in the presence of T cells. Interestingly, *in vitro* analysis demonstrated that crosslinking between tumor cells and T cells for at least 24 h was needed to achieve T-cell activation and development of antitumor activities. Early clinical evaluations demonstrated that the above mouse bispecific antibody also exhibited significant antitumor activity *in vivo* with reasonably acceptable side effects such as mild to moderate fever, nausea, emesis, and fatigue.[121,122] Although an antimouse antibody response was indeed measured near the end of the treatment, there was no indication that it limited the efficacy of the therapy. An improvement in potency was also observed when the peripheral blood mononuclear cells were costimulated by simultaneous treatment with an anti-FR–anti-CD28 bispecific antibody. Although FA-mediated retargeting of T cells to tumors was not specifically examined in these studies, the data nevertheless support the concept of exploiting the overexpression of FR on tumor cells to promote specific immune-cell recognition of neoplastic tissue.

There are still a number of immunotherapy strategies for which applications of folate-mediated targeting have not yet been explored. One such strategy involves linking FA to a superantigen for the purpose of concentrating the superantigen in the cancer tissue. Superantigens are peculiar in that they directly activate a substantial fraction of T cells without the need for presentation in the context of a major histocompatibility complex. Consequently, T cells migrating through the cancer tissue would be rapidly activated even though they may normally not recognize any antigen as foreign in the tumor mass. Activated T cells may then proliferate, release chemokines to invite in other immune cells, and engage in a number of cytotoxic activities that can lead to tumor-cell killing. By focusing these activities in the tumor, the chances of host rejection of the tumor are greatly heightened. Other antigens that might warrant similar evaluation include muramyldipeptide and derivatives/fragments of bacterial lipopolysaccharide.

XIII. FOLATE-TARGETED ENZYME PRODRUG THERAPY

Another approach for improving the tumor selectivity of chemotherapy, termed antibody directed enzyme prodrug therapy (ADEPT), has traditionally combined antibody targeting with enzyme catalyzed prodrug activation. In this strategy, an enzyme-

monoclonal antibody conjugate is administered and allowed to accumulate in the antigen-expressing tissue (e.g., tumor). A nontoxic prodrug is then injected; on contact with the enzyme, the prodrug is converted to its cytotoxic form, that in turn kills the tumor.[123] We have recently adapted this approach for use with FA in place of the monoclonal antibody. In our studies, penicillin-V amidase, a fungal enzyme known to hydrolyze the prodrug, doxorubicin-N-p-hydroxyphenoxyacetamide (DPO) to free doxorubicin, was conjugated to FA and tested for cytotoxicity *in vitro*. Although the potency of the DPO prodrug towards KB cells pretreated with FA–penicillin-V amidase was found to be comparable to that of active free doxorubicin (Lu, J. and Low, P. S., personal observations) the FA-directed enzyme prodrug combination exhibited no toxicity towards A549 cells, an FR-negative cell line. It will be important now to test the folate-targeted enzyme–prodrug approach *in vivo*, where factors such as tumor selectivity and immunogenicity can be more accurately assessed.

XIV. CONCLUSIONS

The folate receptor is significantly upregulated in a large fraction of both solid and hematopoietic human cancers. Conjugates of FA linked to virtually any molecule or molecular complex of diameter <150 nm will bind to the receptor with high affinity ($K_D \leq 10^{-9}$ M) and enter the cells by receptor-mediated endocytosis. Since the same FA conjugates do not bind to FR negative cells even though they express the reduced folate carrier, the conjugates display significant selectivity for cancer cells *in vivo*. This selectivity allows for the possible tumor-specific targeting of imaging agents, genes, antisense oligonucleotides, low-molecular-weight drugs, cytotoxic proteins, radiotherapeutic agents, enzymes for use in prodrug therapies, and immunotherapeutic agents.

Folic acid outperforms most monoclonal antibodies as a tumor-selective targeting agent for many reasons. As noted in Table 5, folic acid is much smaller (Mr ~441) than monoclonal antibodies (Mr ~160,000) and hence can penetrate tumor tissues much more rapidly and completely. FA also exhibits higher affinity for its receptor (K_D ~100 pM) than most antibody–antigen complexes. Although the FR is only one of several well-characterized tumor-associated proteins, its distribution appears to be more widespread (e.g., ovarian, breast, brain, renal, lung, head and neck, endometrial, and some colon cancers, as well as leukemias of myeloid lineage) than other tumor-selective antigens, suggesting it may serve as a more general targeting ligand. Folate is also nonimmunogenic, whereas even humanized monoclonal antibodies can elicit a neutralizing immune response. Folic acid is relatively easy to conjugate to a wide variety of molecules and molecular complexes, whereas antibody conjugation can be nonselective and relatively inefficient. The FR is apparently able to recycle many times and deliver multiple folate conjugates per cell, whereas no tumor-specific antigens have been shown to unload their antibody conjugates inside the cell and return to the

TABLE 5
Comparison of the Properties of FA and Monoclonal Antibodies/Protein Ligands as Tumor Delivery Agents

	Folic acid	Antibody/Protein
Molecular weight	441	160,000/variable
Tumor permeability	High	Low
K_D for cell-surface receptor	10^{-10} M	10^{-10} M to 10^{-6} M
Immunogenicity	Low	Low to high
Conjugation chemistry	Easy	Difficult
Receptor recycles	Yes	No
Stability to acids/bases/solvents	High	Low
Stability during storage	High	Variable
Lysosomal deposition	Low	High
Toxicity of targeting ligand	Low	Variable
Cost	Low	High

cell surface for more. FA is stable to mild acids/bases and a variety of solvents, temperatures, and storage conditions, whereas antibodies must be handled carefully to avoid their denaturation. Folic acid is cheap to procure, whereas monoclonal antibodies are expensive to produce. And folic acid is intrinsically nontoxic, whereas some monoclonal antibodies can alter cell function. In short, for many applications, FA constitutes an attractive alternative to antibodies and other proteins/peptides as a targeting ligand. Clearly, more animal and human clinical studies must be conducted before the usefulness of folic acid can be accurately evaluated; however, the results from many labs to date all point to the fact that it will soon find an important niche in the diagnosis and/or treatment of cancer.

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